

The effects of microgravity on the development of surface righting in rats

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The active interaction of neonatal animals with their environment has been shown to be a decisive factor in the postnatal development of sensory systems, which demonstrates a critical period in their maturation. The direct demonstration of such a dependence on the rearing environment has not been demonstrated for motor system function. Nor has the role of gravity in mammalian motor system development been investigated. Here we report the results of two space flight missions examining the effect of removing gravity on the development of surface righting. Since the essential stimulus that drives this synergy, gravitation, was missing, righting did not occur while the animals were in the microgravity environment. We hypothesize that this absence of contextual motor experience arrested the maturation of the motor tactics for surface righting. Such effects were permanent in rats spending 16 days (from postnatal day (P), P14 to P30), but were transient in animals spending nine days (from P15 to P24) in microgravity. Thus, active, contextual interaction with the environment during a critical period of development is necessary for the postnatal maturation of motor tactics as exemplified by surface righting, and such events must occur within a particular time period. Further, Earth's gravitational field is not assumed by the developing motor system. Rather, postnatal motor system development is appropriate to the gravitational field in which the animal is reared.

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It is presently well documented that nervous system development is influenced by the interaction of young animals with their environment, and that specific age-dependent experiences are required for normal development in sensory systems (Katz & Shatz, 1996). Such an experiential prerequisite was first shown by Hubel and Wiesel in the visual system (Wiesel & Hubel, 1963, 1965). These classical experiments, using monocular eye closure, demonstrated that a 'period of susceptibility' exists during which specific environmental inputs are necessary for nervous system maturation (Hubel & Wiesel, 1970; Wiesel, 1982). These sensitive periods are called 'critical periods' of development when the effects are long lasting (Hubel & Wiesel, 1970).

Much effort has been devoted to understanding the mechanisms underlying such activity-dependent development. Most of these studies have been implemented in sensory systems where visual, auditory, somatosensory, olfactory, or other input has been modified, restricted, or eliminated during postnatal development (Meisami, 1978; Conlee & Parks, 1981; Gray

et al. 1982; Fox, 1992; Schlaggar *et al.* 1993; O'Leary *et al.* 1994; Penn & Shatz, 1999; Zou *et al.* 2004). Although it is difficult to eliminate or restrict vestibular input, studies of otoconia-deficient mice have shown that the development of the vestibular system is dependent on afferent activity (Trune & Lim, 1983; Kozel *et al.* 1998).

The present study addresses the role of experience in the postnatal development of motor behaviour. In the past, manipulation of motor systems has involved invasive paradigms (Sheard *et al.* 1984; Lowrie *et al.* 1987; O'Hanlon & Lowrie, 1993) or pharmacological stimulation (Sanusi *et al.* 1998) that make the interpretation of research findings ambiguous. In our search for a non-invasive approach to altering motor experience, we explored the possibility of removing gravity. We reasoned that since evolution took place in the presence of gravity, this parameter is expected to be present and thus incorporated into the control, by the nervous system, of the movement of each joint of the articulated skeleton (Roberts, 1967). Thus, the presence of gravity may be as fundamental to the development of posture and movement as images are

to the development of vision. Thus, we modified the load imposed on articulated joints by gravity to find whether development of motor function was indeed dependent on experience, and whether 'critical periods' were involved. In particular, we asked if the presence of a gravitational field is necessary for the proper development of motor function.

First, we adapted neonates to a non-invasive model, tail suspension, used for simulating weightlessness in adult rats (Fitts *et al.* 1986). (In this paradigm animals are suspended by their tails to lift their hindlimbs off the ground.) By unloading the hindlimbs of neonatal rats, tail suspension modified the effects of gravity on the developing neuromuscular system. In our tail suspension studies, motor function before postnatal day (P) 15 was evaluated as the ability of the neonates to maintain their balance in the water and to swim. In older animals, locomotion and air righting were evaluated. Using these measures, we identified P8 to P13 as a sensitive period of development because tail suspension significantly increased swimming stroke duration in animals suspended during this period. Swimming style was also affected and the balance of suspended animals in the water was poor. This was most marked after three days of suspension (P11) when the animals could not keep their heads above the water and twisted about their longitudinal axis and sank. However, because these changes were reversed after suspension was ended, this was identified as a 'sensitive' rather than a 'critical' period of development. We identified P13 to P31 as a critical period for the development of motor skills because animals suspended during this period showed changes in locomotion that persisted unchanged for the 30 days after suspension they were studied (Walton *et al.* 1992; Walton, 1998). These findings provided the basis for the current space flight experiments.

In the first space flight experiment, neonatal rats experienced the microgravity of low earth orbit for nine days from P15 to P24. In the second experiment, the space shuttle flight lasted for 16 days and the animals were in microgravity from P14 to P30. Microgravity has several advantages over tail suspension in this type of study. In microgravity the entire animal, not only the hindlimbs, experiences unloading; the animal's posture is not restricted; and the vestibular system, which plays an important role in motor function, is deprived of its fundamental gravitational input.

Several measures of motor function were evaluated in animals flown in space and those remaining on the ground including swimming (Walton *et al.* 2005) and free walking. Here, we report on the development of surface, or contact, righting. During ontogeny, surface righting reactions are among the earliest global motor behaviours executed by terrestrial vertebrates. They are also among the earliest defined in comparative neurology (Magnus, 1924). This behaviour is particularly suited to a study of motor system development because; (a) complex sensory-motor

organization is required for the efficient rotation from a supine to a prone position, and (b) this motor skill gradually appears in its mature form over the first three postnatal weeks (Altman & Sudarshan, 1975; Markus & Petit, 1987; Pellis *et al.* 1991).

Based on our tail suspension studies (Walton *et al.* 1992), we hypothesized that the development of surface righting would be most sensitive to microgravity in flight animals launched on P8, but that this influence would be permanent only in the animals that remained in microgravity until P30. This paper reports our findings in animals launched on P14 or P15. An abstract of this work has appeared (Harding *et al.* 1999).

Methods

The experimental design compares motor function in young rats launched into low-earth orbit with age-matched control animals that remained on earth. The data set comprises measurements of surface righting in animals from two space shuttle flights: Neurolab (STS-90), a 16-day mission that included in-flight experiments; and National Institutes of Health Rodent Experiment 3 (NIH-R3, STS-72), a 9-day mission that did not include in-flight experiments. These are called the 16-day and the 9-day mission, respectively. They remain the only missions in which neonatal mammals experienced space flight.

Designation of days

Although only the flight animals were actually launched into space, we also use the term 'launched' to designate the first day of the experimental period for the ground control animals. Likewise, we use the term 'landed' to indicate both the day the flight animals returned to earth and the day the control animals were transferred to standard cages and returned to the general housing area of the Kennedy Space Center life sciences support facility. For both the animals flown in space and the those remaining on the ground, the day of the launch is also the first flight day while the last flight day, the day of landing, is called return day 0 (R0).

Animals

Sprague-Dawley dams and litters were obtained from Taconic Farms (Germantown, NY, USA). Litters with 7 female (16-day mission) or 10 male and female (9-day mission) neonates were delivered to Kennedy Space Center when the neonates were P7 (± 12 h). Upon arrival at the life sciences support facility at the Kennedy Space Center, each dam and litter was individually housed in a standard vivarium cage. The animals were put on the same diet as during the subsequent space flight (food bars from Teklad Diet, American Institute of Baking). The specific pathogen-free status of each dam was verified.

Table 1. Ages at launch and landing and the number of animals in each experimental group

Mission	Age		Number of animals			
	At launch	At landing	Flight group	Control groups		
				Flight	Vivarium	Launch
9-day	P15	P24	10	10	—	—
16-day	P14	P30	8	8	8	8

Selection. Animals were chosen for the experimental groups after a two-step process: (1) dams that did not accept the food bars (both missions) or drink from the automatic watering spouts (16-day mission only) within the first 24 h of arriving at the Kennedy Space Center were removed from the study; (2) two days before the scheduled launch, litters were chosen based on dam weight gain, litter weight gain, and food and water consumption over the last four days. For the 9-day mission the interaction of the dams with the neonates was also considered in selecting the animals. That is, only dams that immediately returned to the litter after being separated were eligible for selection.

Groups. The selected animals were divided into four groups: (1) the animals flown in space (flight animals); (2) age-matched ground control group housed in flight-like cages; (3) age-matched ground control group housed in standard vivarium cages; and (4) P14 control animals housed in standard vivarium cages (Table 1). These animals were used to establish baseline values for motor function in P14 animals. The flight and age-matched control animals could not be studied on the day of launch since they were already in the space shuttle or the ground control holding room.

Experiments were carried out aboard the space shuttle Columbia, at the Kennedy Space Center life sciences support facility, and at the New York University School of Medicine. Local government authorities and the National Aeronautics and Space Administration (NASA) approved all experimental procedures. The animals were cared for according to NIH, NASA, and New York University School of Medicine guidelines. The animals were weighed before the flight, on the day of landing, and during the post-flight experimental period. The animals ate and gained weight normally during the flight and during the postflight experimental period (see Fig. 9). At the end of the post-flight experimental period the 16-day animals were used for anatomical studies after humane killing (DeFeliipe *et al.* 1999). At the end of the postflight experimental period, the 9-day animals and the P14 control animals were killed with an overdose of phenobarbital according to NIH, NASA, and New York University School of Medicine guidelines. When the animals were weaned, the dams were also killed with an overdose of phenobarbital.

Housing. Animals were loaded into flight, flight-like or vivarium cages the day before the scheduled launch. The cages used for the flight and flight-cage ground control animals were called animal enclosure modules and held two separate litters. A 9-day mission flight cage during cage unloading is shown in Fig. 1A. The animal enclosure modules used in the two missions were similar in design. For the 16-day mission 546 cm³ was available to each animal (~4 916 cm³ for 8 neonates + 1 dam), for the 9-day mission, 6 555 cm³ was available to each animal (~590 cm³ for 10 neonates + 1 dam). Thus although the cage sizes were not the same, the density of animals did not differ significantly. The other control animals were housed in standard vivarium cages (~15 000 cm³). All control animals were housed in designated ground control holding rooms in the life science support facility, from the scheduled day of launch to the day of landing.

The 9-day mission launched on schedule. The 16-day mission launched after a 24 h delay during which the animals remained in the space shuttle (flight animals) or ground control holding room. Within 3–4 h of landing the cages were removed from the space shuttle or ground control rooms and brought to a 'portable clean room' within the life science support facility. Here the animals were removed from the cages (see Fig. 1A) and inspected by a veterinarian. In both missions all the animals were judged to be in good health and were distributed to the research teams.

Procedures and data analysis

In-flight procedures. During the 16-day mission, animals were tested inside a general-purpose workstation in the shuttle laboratory (flight animals) or in the life science support facility (ground control animals housed in flight-like cages). An 'animal walking apparatus' was constructed for these experiments (Fig. 1B). This comprised a rectangular platform with a foam surface on one side and a wire grid on the other. In the surface righting experiments, the foam surface was used. There were two metal bars above and two metal bars below the platform. For each trial, an animal was placed supine on the foam surface with its head straight or tilted ~90° about the roll axis. When an animal was immobile and relaxed, it was quickly released. This procedure was repeated 5–10 times for each head position. Two video cameras (TEAC, Sekai, 60 frames s⁻¹ (fps)), fixed at right angles to the animal walking apparatus, recorded the animal's movements. These procedures were carried out on flight day 6 (five animals), and the day of landing (eight animals). Post-flight procedures (see below) were also carried out on the day of landing.

Postflight procedures. The protocol that was used after landing differed from that described above in four ways: (1) the animal's head was held straight before release; (2)

the animal was placed on a large (76 cm by 76 cm) foam pad; (3) more trials were carried out for each rat (up to 20); (4) a single high-speed camera recorded the animal's movements (NAC, 200 or 500 fps). Surface righting was tested in the life science support facility on the day of landing and 1, 3, 5, 7, 10 and 23 days after landing, and at New York University School of Medicine on 110 and 124 days after landing. After the 9-day mission, surface righting was tested in the life science support facility on the day of landing and the seven following days (R0–R7).

Data analysis. The posture of the animal during surface righting, and the time required to complete the movement were evaluated in flight and ground control animals. The posture during each turn was assigned to one of three categories previously described by Pellis (Pellis

et al. 1991). (1) Axial righting is the adult form of righting. The animal rights by cephalocaudal axial rotation (Fig. 2A). Rotations triggered by snout contact with the ground were included in this category although this 'trigeminal-on-head' righting is considered by some to be behaviourally distinct from axial righting (Pellis, 1996). (2) Corkscrew is an intermediate form of righting. The head and forelimbs first rotate in one direction while the hindlimbs rotate in the opposite direction (Fig. 2B). The hindlimbs then rotate in the same direction as the head and forelimbs to complete the turn. (3) U-posture righting is seen in the youngest animals. The animal ventroflexes with quadrupedal extension before rotating its head and body in the same direction (Fig. 2C). For each animal, the occurrence of each posture was expressed as a percentage of the total number of trials for that day. These categorical data were expressed as the group mean of the individual animal means.

Three values for surface righting times were determined using frame-by-frame analysis: When the animal was free to move (Start), when the head rotated 90 deg (16-day mission) or 180 deg (9-day mission) (Head), and when both hindlimbs touched the ground to complete the movement (Hindlimbs). Using these data,

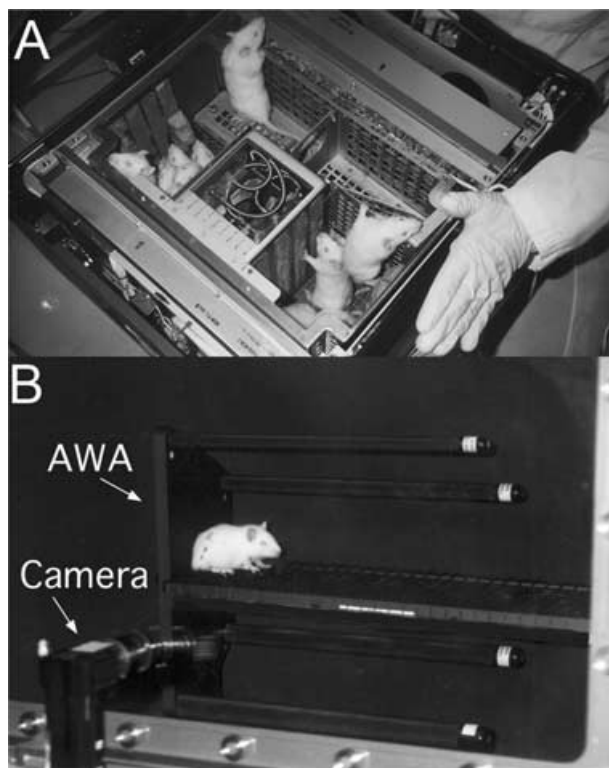


Figure 1. Flight cage and animal walking apparatus

A, similar cage design was used in both missions. Animal enclosure module and animals during cage unloading after the 9-day mission. Note that the animals are rearing and attempting to leave the cage. B, the in-flight and parallel ground control experiments were carried out using an animal walking apparatus (AWA) placed inside a general purpose workstation as shown here. Two cameras, placed outside the workstation, and at right angles to each other, recorded the animal's movements. One camera is shown; the other faced the front of the animal in the photograph. The platform can be rotated 360 deg and is shown with the grid side up. Note the two bars at different distances above and below the platform. This photo was taken on the ground. The animal was about P15. (The joints of the animal were marked for other experiments.)

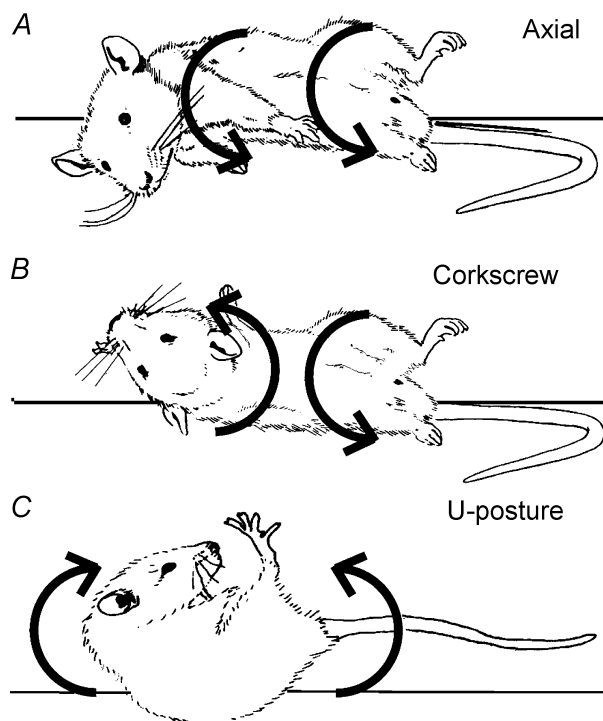


Figure 2. Surface righting tactics

A, cephalocaudal axial rotation is characteristic of righting in adult animals. B, in corkscrew righting the head and forelimbs first rotate in one direction while the hindlimbs rotate in the opposite direction. The hindquarters then rotate in the same direction as the head and forelimbs. C, in U-posture righting ventroflexion with quadrupedal extension precedes head and body rotation in the same direction.

the time to turn the head (Head–Start), the hind-quarters (Hindlimbs–Head), and to complete righting (Hindlimbs–Start) were calculated. Only trials in which the entire turn could be seen clearly and the movement was not impeded were measured.

Statistical analysis was carried out using one-way ANOVA with Scheffe's *post hoc* test (for more than one nominal independent variable), or using Student's unpaired *t* test (for one nominal independent variable). Fisher's *r* to *z* was used to determine significance of correlation coefficients (StatView 5.0, SAS Institute Inc.). A level of $P < 0.05$ was considered to be statistically significant. From 12 to 15 trials per animal per day were included in our analysis.

Results

We have found that the presence of a gravitational field is necessary for the postnatal development of surface righting in neonatal rats. The data from the 16-day and 9-day missions are presented together. The results are organized to answer six basic questions.

Does surface righting occur in microgravity?

Access to a microgravity environment provided a unique opportunity to study surface righting because the contribution of the gravitational sensors could be separated from tactile and somatosensory signals. We found that the presence of a gravitational field is necessary for surface righting to be elicited in young rats. Indeed, no righting reactions were elicited during the in-flight trials when the animals were released with the head straight (0 of 13 trials). Neck-on-body righting was not elicited when the animals were held supine with the head rotated about the roll axis (0 of 12 trials). Indeed, rather than rotating the body to become aligned with the head as in the control animals, when they were in space, the animals rotated the head to align it with the body. When animals were released in microgravity, they exhibited a rapid and sustained extension of all four limbs. This posture was maintained until the limbs touched a surface. Most often, this surface was provided by one of the bars located above the animal walking apparatus platform (Fig. 1B). In contrast, the age- and cage-matched ground control animals executed prompt righting on all the trials (21 of 21 trials from each position). In answer to the first question: surface righting does not occur in microgravity.

Is surface righting similar in flight and control animals on the day of landing?

The strategy the nervous system uses to achieve a particular goal, e.g. being upright, is couched in general terms such

as 'move from supine to prone'. The exact movements used to achieve this goal, the motor tactics, vary in neonatal rats over the first postnatal weeks. The normal development of surface righting tactics follows an overlapping progression from U-posture, to corkscrew, to axial righting as described by Pellis (Pellis *et al.* 1991) (see Fig. 2).

Launch. During the 16-day mission, we tested a group of ground control animals that were the same age as the flight and control animals at launch (P14). These animals provided a baseline for the surface righting tactics and speed that were measured after landing. (These P14 data are included in Figs 3, 5 and 7A)

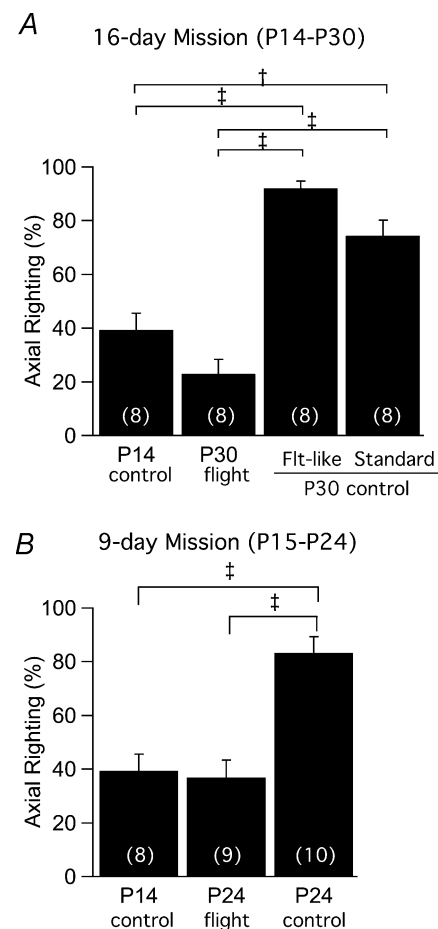


Figure 3. Percentage of trials utilizing axial surface righting at launch into low earth orbit and within hours of landing

A, 16-day mission. 14-day-old control animals, animals flown in space (P30 flight), control animals housed in flight-like cages (Flt-like P30 control) or in standard vivarium cages (Standard, P30 control) on the day of landing. Note that there were no differences between P14 (launch age) and flight animals on the day of landing although both these groups differed from P30 control animals ($n = 8$ animals per group). B, 9-day mission. P14 control animals (same as A), P24 flight, and P24 control animals on the day of landing show the same pattern as for the 16-day mission. Results are mean \pm S.E.M. $\dagger P < 0.001$, $\ddagger P < 0.0001$, ANOVA; $n = 9$, P24 flight; $n = 10$, P24 control animals.

Landing. Both control and flight animals were able to right themselves on the first trial after landing. However, the righting tactics used by the flight animals were more similar to those of the P14 animals than to the age-matched ground controls. This is shown unambiguously in Fig. 3A for the 16-day mission and Fig. 3B for the 9-day mission. The mean percentage of trials in which axial righting was used is plotted for P14 (16-day mission launch) and on the day of landing (P30) in flight and control animals housed in flight-like (Flt-like) or standard vivarium cages. In all groups of control animals, the percentage of trials using axial tactics more than doubled between P14 and P30. However, in flight animals from both missions this value was essentially identical to that at P14. Since this was seen in animals on both missions, it was independent of mission duration and age at landing.

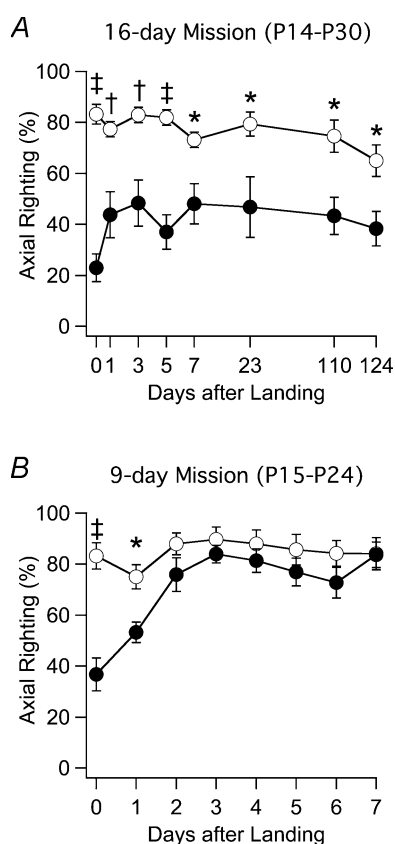


Figure 4. Axial righting in flight and control animals after landing: 16-day mission

There were no changes in the percentage of axial righting in flight (●) or control (○) animals between the day of (day 0) and 124 days after landing (P154) ($n = 13$ –16 control, $n = 8$ flight animals except day 3 $n = 7$; day 23, $n = 4$). **B**, 9-day mission. Axial righting increased in flight animals (●) between the day of landing and 2 days later when they reached control levels. ($n = 8$ –10 control, $n = 8$ –11 flight animals). Values are mean \pm S.E.M. * $P < 0.05$, † $P < 0.001$, ‡ $P < 0.0001$, unpaired t test. Each data point represents 12–15 individual trials $\text{rat}^{-1} \text{ day}^{-1}$. In A the days are discontinuous, however, symbols are connected for clarity.

As shown in Fig. 3A, there were no significant differences in surface righting between the animals housed in flight-like cages and those housed in standard vivarium cages during the 16-day mission. For this reason, the data from both groups are combined into one ground control group in the remaining figures. As illustrated in Fig. 3, the answer to the second question is: surface righting tactics differ in flight and control animals on the day of landing.

Are the righting characteristics seen on landing day maintained throughout the postflight period?

Axial righting. We next evaluated the frequency of axial righting during the postflight period to find if the development of surface righting tactics, interrupted by removing gravity, resumed after landing. The mean percentage of axial righting for control (○) and flight (●) animals is plotted as a function of days after landing for animals on the 16-day and the 9-day mission in Fig. 4A and B, respectively. In the flight animals on the 16-day mission (Fig. 4A, ●) the mean percentage of axial righting did not change significantly throughout the post-flight testing period (return day 0–124; P30 to P154), and remained significantly lower than in the control animals. In contrast, in the animals flown in space for 9-days (Fig. 4B, ●) there was a sharp increase in axial righting after landing reaching control levels two days after landing (P26). There were no further significant changes in this parameter.

Non-axial righting. The histograms in Fig. 5 illustrate the mean occurrence of each righting tactic for animals on the 16-day (Fig. 5A and C) and the 9-day (Fig. 5B and D) mission. The values are plotted as a function of age for control animals and a function of days after landing for flight animals (P14 is included for comparison). As shown in the first column of each panel, corkscrew righting (green pattern) was the predominant non-axial tactic at P14. Two new righting tactics were observed for the first time on the day of landing. A form of U-posture righting in which ventroflexion was so extreme that the animals sat upright (called L-posture righting; diagonal red lines). This righting tactic was seen in all four animals groups. U-posture followed by corkscrew righting (called U and C (U & C) righting, short red lines) was only seen in animals that had flown in space. This may be seen in a video (see Supplemental material).

Righting was very similar in control animals from both missions (Fig. 5A and B). Between launch and landing there was a significant decrease in the use of corkscrew ($P < 0.05$) in favour of axial righting. However, during the postflight period there were no significant differences in the tactics used by either group of control animals.

When the tactics used on P14 (16-day mission launch) and on landing day were compared, the same pattern was seen in flight animals from the 16-day (Fig. 5C) and the 9-day (Fig. 5D) mission: (1) the occurrence of axial righting was similar at launch and landing; (2) corkscrew righting decreased from launch values ($P < 0.05$); (3) L-posture and U & C righting appeared for the first time; (4) U-posture righting increased from launch values (this was significant in the animals flown in space for 9 days, $P < 0.01$).

After the day of landing there were differences between the two missions. In the animals flown in space for 16 days, tactics incorporating ventroflexion (bars containing red) predominated through to recovery day 23 (R23, Fig. 5C),

and were greater than control values on each test day ($P < 0.01$). After three months of recovery, the dominant non-axial tactic was corkscrew righting (Fig. 5C, last two columns). This was greater than control values ($P < 0.05$, t test). Note that the percentage of trials utilizing axial righting was unchanged. In the animals flown in space for 9 days, tactics incorporating ventroflexion reached control levels by one day after landing, and corkscrew righting reached control levels by two days after landing. There were no further significant changes in the tactics used. There is no single answer to the third question because the persistence of righting tactics after landing is influenced by the age of the animals, the length of the flight, or both factors.



Figure 5. Mean frequency distribution of surface righting tactics

A and B, control animals. Distribution of righting tactics as a function of age for 16-day mission (A) and 9-day mission (B). Note that corkscrew righting (green pattern) predominated on P14 and decreased in favour of axial righting (white) after P30. There were no significant changes during the postflight period in control animals from either mission. C and D, animals flown in space. Data from 16-day (C) and 9-day (D) mission plotted as a function of days after landing (first column P14). Note the appearance of L-posture and U & C righting tactics on the day of landing (day 0) in both missions. C, 16-day mission. In contrast to control animals, there were no differences between P14 and landing (day 0) in flight animals. Righting with ventroflexion (L-, U-posture, U & C, red fills) predominated until day 23. D, 9-day mission. On landing day (day 0) there was a decrease in corkscrew righting with an increase of righting with ventroflexion (L-, U-posture, U & C; red fills) compared to one day before launch ($P < 0.001$). Same number of animals as in Fig. 4; unpaired t test.

Does head position influence righting tactics?

To examine which factors influence righting tactics, on the day of landing we evaluated the effect of head position in the animals from the 16-day mission. The righting tactics used are compared in Fig. 6 for animals held supine with the head straight (open bars) or the rotated about the roll axis (filled bars). In the control animals, rotating the head eliminated U-posture righting ($P < 0.01$) and decreased corkscrew righting ($P < 0.01$) in favour of axial righting (Fig. 6A). In the animals flown in space (Fig. 6B) head rotation increased axial ($P < 0.05$) and corkscrew ($P < 0.01$) righting, decreased U- and L-posture righting, and eliminated U & C righting. Significant differences remained between flight and ground control animals in the proportion of axial ($P < 0.0001$), corkscrew ($P < 0.05$), and U-posture ($P < 0.0001$) righting from the head-rotated position (t test). These data indicate that initial head position influences which righting tactic is used.

Does microgravity differentially influence the development of surface righting tactics and speed as tested on landing day?

Animals flown in space versus ground control animals.

To find if righting speed was influenced by microgravity we compared axial, corkscrew and U-posture righting times at launch (Fig. 7A, striped bars) with those on the day of landing in flight (Fig. 7A, filled bars) and control animals (Fig. 7A, open bars) from the 16-day mission. Righting time decreased from launch (P14) values in both flight and control animals for all three tactics. Nevertheless, axial ($P < 0.001$) and corkscrew ($P < 0.05$) righting was slower in flight than in control animals at landing (Fig. 7A). Data pooled over the postflight period (return day 1 to 124) show that corkscrew righting was faster in flight animals (Fig. 7B) while there was no difference for the other tactics.

Righting tactics. As shown in Fig. 7 for the 16-day mission, the time required for righting varied with

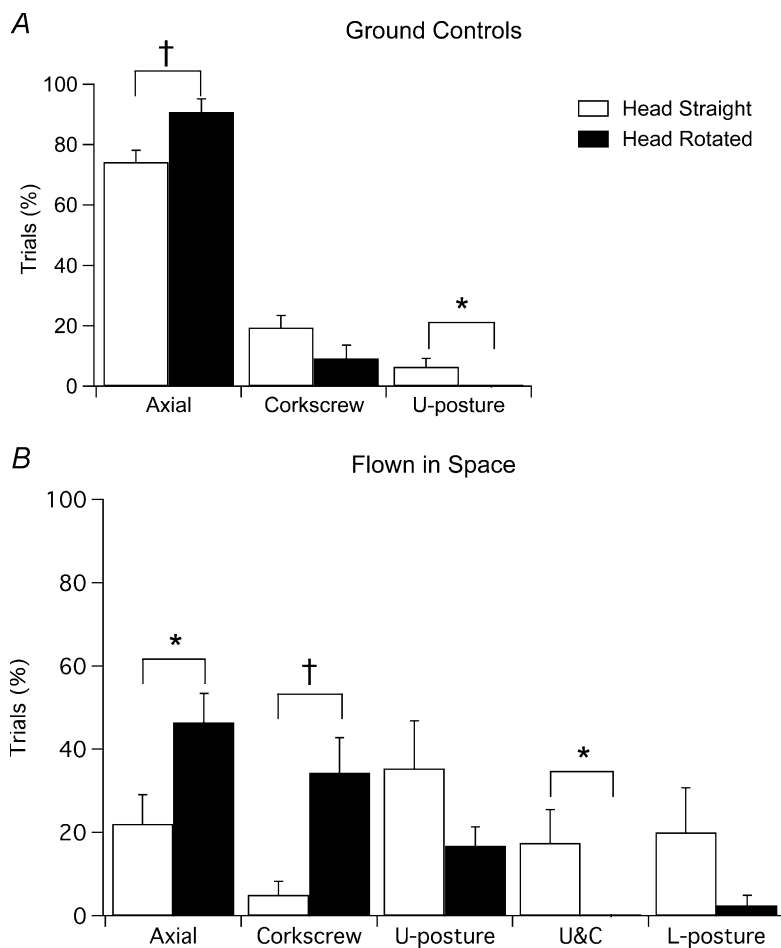


Figure 6. Effect of head position on surface righting tactics in P30 animals on landing day for the 16-day mission

A, control animals. When released from the head-rotated position (filled bars), axial righting increased, corkscrew righting decreased and U-posture righting was eliminated. B, animals flown in space. Head rotation eliminated U & C righting and reduced U- and L-posture righting in favour of increased corkscrew ($P < 0.01$) and increased axial ($P < 0.05$) righting. This figure incorporates trials carried out using the in-flight protocol. Values are means \pm S.E.M., * $P < 0.05$, † $P < 0.001$, unpaired t test; $n = 8$ flight, $n = 11$ control.

the tactic utilized. The following sequence was seen in P14 and P30 control animals (Fig. 7A), and in flight- and age-matched ground control animals over the postflight period (Fig. 7B, Table 2): axial < corkscrew = U-posture < U & C < L-posture ($P < 0.05$). In contrast, there was no difference in speed among axial, corkscrew, and U-posture righting (Fig. 7A, filled bars) in flight animals on the day of landing. Righting times for all tactics decreased after landing, but axial righting decreased more than the others. As a result, axial righting was faster than U-posture righting on the first day after landing ($P < 0.001$, ANOVA, not shown) and axial was faster than corkscrew righting on the fifth day after landing ($P < 0.005$, ANOVA, not shown). Note that in all groups of animals corkscrew and U-posture righting took the same amount of time. Table 2 summarizes the significance of differences between times for righting tactics during the postflight period.

Axial righting. Since axial righting was the most prevalent form ($n = 2169$ trials, 62%) it was chosen for detailed analysis. Axial righting was divided into two consecutive movements; head rotation and hindquarter rotation. (The sum of these gives the total righting times which are plotted in Fig. 7.) Head (flight ●, control ○) and hindquarter (flight ■, control □) rotation times are plotted as a function of days after landing in Fig. 8. On the day of

landing, both head and hindquarter rotation was slower in flight than control animals. The majority (76%) of the difference in total axial righting time in Fig. 7A was due to the slow hindquarter rotation. In flight animals there was a 36% decrease in both head and hindquarter righting times ($P < 0.0001$) between landing day and one day later. In control animals there was a 15% increase in the time for hindquarter rotation ($P < 0.05$). After R1 there were occasional significant differences in the head (day 3) or hindquarter (day 10) rotation between flight and control animals, but there were no trends.

In the 9-day mission, axial righting was slower in the flight than in the control animals on landing day (not shown). This was largely (about 70%) due to slow head, rather than hindquarter, rotation in animals flown in space. Control values were reached by the second day after landing.

Individual trials. To find if there was a difference in righting times over the course of one session, we examined individual trials on the first day of testing in P14, P30 ground control, and P30 flight animals. The time required to turn the head, the hindlimbs, and the entire body was evaluated for trials in which head rotation was the first movement (axial and corkscrew righting). The time for head rotation decreased throughout the session in flight animals (correlation coefficient, -0.578 ; Fisher's r to z

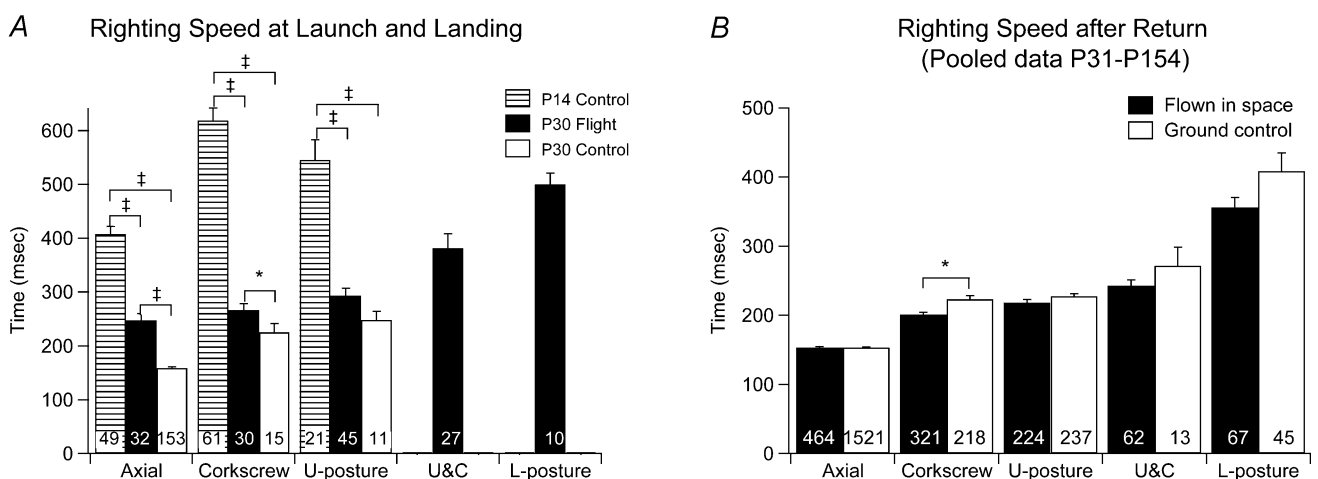


Figure 7. Speed of righting at launch and on landing day after the 16-day mission

A, time required to complete the turn was dependent on tactics in P14 control (horizontal stripes), and P30 control (open bars) animals but not in P30 flight (filled bars) animals. In P14 control animals, axial righting was faster than corkscrew ($P < 0.0001$, ANOVA) or U-posture ($P < 0.01$, ANOVA) righting. In P30 control animals there were differences between each tactic and the next faster one ($P < 0.0001$, ANOVA). In flight animals L-posture ($P < 0.0001$, ANOVA) and U & C ($P < 0.01$, ANOVA) were slower than axial, corkscrew or U-posture that did not differ from each other. L-posture righting was also slower than U & C righting ($P < 0.05$, ANOVA). Righting was slowest before launch (P14) and slower in P30 flight than in P30 control animals on the day of landing. B, mean righting time for flight and age-matched control animals after landing. Note the same dependence of speed on tactic as in A. Data were pooled from R1, R3, R5, R7, R10, R23, R36, R110, R117, R124. Values are mean \pm S.E.M. * $P < 0.01$, † $P < 0.001$, ‡ $P < 0.0001$; ANOVA (>2 factors) or unpaired t test (2 factors). The number of observations is given within each bar.

Table 2. Significant differences in righting time for different tactics, 16-day mission. The fastest tactics are listed first (rows and columns). Data have been pooled for all postflight measurements R0–R154. Note that corkscrew righting is not significantly faster than U-posture righting

Group	Corkscrew		U-posture		U and C		L-posture	
	C	F	C	F	C	F	C	F
<i>n</i>	233	353	248	269	14	89	46	77
Axial	†	†	†	†	†	†	†	†
Corkscrew			—	—	*	†	†	†
U-posture					*	†	†	†
U & C							†	†

C, flight cage and vivarium cage ground controls; F, flight †, $P < 0.0001$; * $P < 0.05$, $n = 1673$ axial control; $n = 496$ axial flight, comparing tactics in columns with tactics in rows.

$P < 0.0001$). This correlation was not seen in P14 or P30 ground control animals. There also was no correlation between trial number and the hindlimb or entire body righting times in any group.

In answer to the fifth question: microgravity had a differential effect on the development of righting tactics and speed. The movement sequence required for righting was not activated when the animals were in microgravity. However, the animals continued to move and their overall movement speed increased while they were in microgravity.

Was the nutritional state of the animals a significant variable in our behavioural findings?

One consideration in carrying out space flight experiments is differentiating the effects of microgravity from other parameters of space flight. This is particularly the case for young animals where nutritional state may be adversely affected. To evaluate this factor, we weighed the animals on the day of landing and during the postflight study. For both missions the weight of the flight animals did not differ significantly from the age-matched ground controls (Fig. 9A and B). To determine if flight and control animals gained weight at the same rate after landing, a straight line was fitted to the weight *versus* days after landing plot for each animal (correlation coefficient 0.99–1) and the slope determined. The mean slope for each group of animals was then calculated. Over the first 30 days after landing, flight and control animals gained weight at the same rate (Fig. 9C and D). We did observe a clear cage effect on the growth of the two groups of 16-day ground control animals. The vivarium animals were heavier than the control animals housed in flight-like cages ($P < 0.05$) on landing day. The answer to the fifth question is: nutritional state, as reflected by both body weight and weight gain did not significantly change, thus it was not a determinant of our behavioural findings.

Other parameters such as light level or temperature were regulated both in ground control and flight conditions, as dictated by animal welfare guidelines. It was only during the short period of launch and landing that such issues as increased vibration and noise common to all space flights could not be controlled. Centrifuge studies simulating the changes in gravitational force during launch and landing showed that these changes alone did not influence surface righting (K.W. personal observation). The only long-term parameters that differ were radiation levels and the reduction in the gravitational field.

Discussion

Comments on space-flight studies

Historically, biological studies concerning the microgravity of low earth orbit have been characterized by limitations that are not typically faced in ground-based studies. These include a small number of animals, the inability to control mission duration, a delay between landing and receipt of the animals by the investigators, and the need to share the animals with other investigators. In addition, the surface area available to the animals for locomotion is increased in microgravity, since animals move about the sides and top of the cage as well as the earth-defined cage bottom. The present study has overcome many of these shortcomings. Young animals have less mass than adults, allowing us to increase the number of subjects, and animals were received within hours of the shuttle landing. We had the rare opportunity of two related

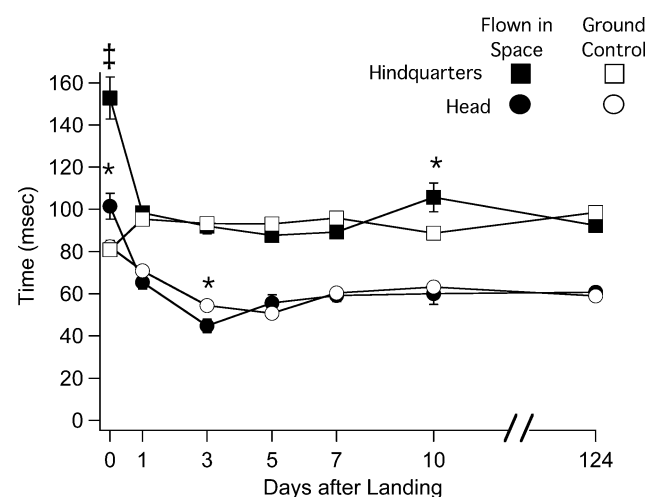


Figure 8. Speed of axial surface righting over time in 16-day mission animals

Hindquarter (flight, ■; control □) and head (flight, ●; control ○) rotation times were longer in flight animals on landing day (day 0), but reached control levels one day after landing. Differences were seen later during the postflight period as indicated. Values are mean \pm S.E.M. * $P < 0.01$, † $P < 0.0001$; unpaired *t* test; flight, $n = 33$ –60 observations except 16 on day 10; control $n = 137$ –177.)

missions thus allowing us to compare missions of different duration. The major factors beyond our control in the present missions were the different ages of the animals at launch (P14 and P15) and a limitation on the postflight study period after the 9-day mission to 7 days. However, our results are so unambiguous as to conclude that for this type of study the advantages of space flight far outweigh its limitations.

Major conclusions

Our findings suggest that the maturation of surface righting occurs through activity-dependent sensory-motor circuit optimization, and this involves the proper dynamic interaction of utricular- and saccular-dependent vestibulocolic and vestibulospinal reflexes to ensure proper ipsilateral head, shoulder, and hindquarter rotation. Further, we hypothesize that the presence of gravity is a prerequisite and that the primary effect of removing this stimulus occurs at the level of the central nervous system (CNS) rather than at the peripheral receptor system, muscles, or bones.

The central findings supporting these conclusions are: (1) surface righting synergy was not activated under microgravity conditions; (2) in the absence of the appropriate gravitational stimulus, other, simpler synergies were implemented that allowed rapid intentional movements without the prodromic postural rearrangement predicated by earth's gravitational field (Fig. 3); (3) the development of surface righting resumed

in P24 animals after a 9-day space flight, but not in P30 animals after a 16-day space flight (Figs 4 and 5).

Surface righting: a synergy to counter gravity

The development of surface righting over the first three weeks of life (Pellis *et al.* 1991) exemplifies the post-natal optimization of motor skills to achieve optimal execution (energy conservation and speed) typical of adult movements (Bernstein, 1967; Abitbol, 1988). Rats are capable of righting themselves from the supine position at birth. Indeed, rotation is an important component of the motor strategy neonates use to reach the dam's nipple (Eilam & Smotherman, 1998). There is an overlapping progression from non-axial to axial righting with age: U-posture righting, predominating at birth, is first lost in favour of corkscrew righting (P0 to P12), which is then lost in favour of axial righting (P10–P25). There is a sharp increase in axial righting near P10, this tactic predominates by P15–P16 and continues to increase during the second postnatal week (Pellis *et al.* 1991) (Fig. 5A and B).

We have shown that an important element in determining surface righting tactics is whether the first movement is head rotation or ventroflexion. Indeed, head rotation eliminated tactics utilizing ventroflexion in favour of axial and corkscrew righting (Fig. 6), following the same sequence as seen during normal development.

Through the in-flight trials we have shown for the first time that surface righting in rats requires appropriate otolithic input. The tactile and somatosensory clues

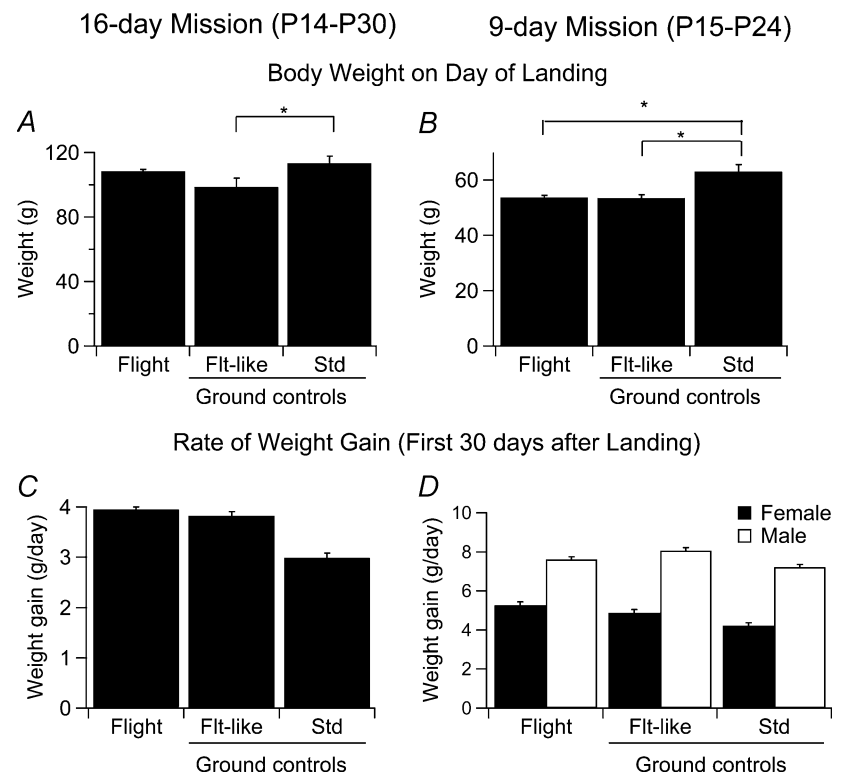


Figure 9. Mean body weight on the day of landing and weight gain after landing

A and B, on the day of landing there were no differences in the weight of flight, animals and control animals housed in flight-like cages (Flt-like) from either mission. Control animals housed in standard vivarium cages were heavier than other groups as indicated. C and D, there were no statistically significant differences in the rate of weight gain after landing. Note that male animals gained weight at a faster rate than female animals (D). (16-day mission animals were all female.) Results are mean \pm s.e.m., * $P \leq 0.05$, ANOVA; animals A, $n = 8$; B, $n = 10$ –11; C, $n = 7$ –8; D, $n = 5$ –8 female, $n = 3$ –5 male.)

present when an animal was 'placed on its back' in space were insufficient to elicit righting. Under such conditions, even when these stimuli were supplemented by head rotation about the roll axis, righting movements were not elicited. It is clear therefore that the vestibulocolic (vestibular-on-head) and vestibulospinal (neck-on-body) reflexes (Magnus, 1924) were not activated. In contrast, when the animal was released from the head-rotated position, the head moved to become realigned with the body. This head movement, opposite to that needed for righting, demonstrates that: (1) the intact cervicocolic stretch reflex predominated over other stimuli in microgravity; (2) colic input to vestibular circuits elicits appropriate responses; (3) animals are able to execute a well-defined movement without having to organize their postural system as is necessary in the presence of gravity.

Upon landing it was clear that the maturation of righting tactic normally seen after P14 and P15 (Pellis *et al.* 1991) did not occur while the animals were flown in space. Indeed, the percentage of axial righting immediately post-flight was indistinguishable from preflight values (Fig. 3). In addition, there was an increased use of righting with ventroflexion on the day of landing in flight animals from both missions (Fig. 5). This continued for at least three weeks in animals from the 16-day mission and could be viewed as developmental regression since such righting predominates at birth (Pellis *et al.* 1991).

Activity-dependent circuit optimization

In the classical description of air righting, head rotation is elicited by otolithic stimulation; shoulder rotation is then recruited by head rotation, and hindquarter rotation is, in turn, recruited by the asymmetrical position of the forequarters (Magnus, 1924). This sequence occurs in the rat, although the need for a cervical reflex to recruit shoulder rotation has been questioned (Pellis, 1996). According to the classical description, axial and corkscrew righting may be considered as legitimate righting tactics while those involving ventroflexion fall outside this category. Ventroflexion occurs when the mid-line musculature is activated in the absence of head or shoulder rotation. It is elicited, at least in part, by direct vestibular activation since this movement was not seen in microgravity. However, ventroflexion is poorly matched with the motor intention of being prone and may result from the undifferentiated recruitment of motoneurone pools by vestibular and other input.

The relative stability of axial righting frequency after space flight (Fig. 5C and D) suggests that once these circuits are established they are quite robust and are not subject to change. This is in contrast to those underlying other righting tactics, which seem to be more sensitive to lack of use. This is a reasonable hypothesis since neuronal circuits activated during axial righting will serve

the animal throughout its lifetime, while those activated during non-axial righting are transient and only activated by otolithic input in the first weeks of life.

The increased speed of righting in flight animals between launch and landing support the continued development in microgravity of neuronal circuits that would be activated during surface righting on the ground (Fig. 7). This is consistent with the fact that these circuits take part in other movements. The slow righting on the first day of testing was probably due to a lack of utilization of these circuits in the context of righting, rather than a delay in development of cellular processes underlying movement speed such as myelination. This interpretation is supported by two observations: (1) on the first day of testing, there was a negative correlation between trial number and righting speed in flight but not control animals. Thus, as the flight animals gained motor experience their performance improved (contextual circuit optimization); (2) flight animals from both missions reached control levels by the second day of testing.

These observations suggest that circuit maturation at the spinal cord level plays a role in the acquisition of mature surface righting tactics. Thus, surface righting maturation occurs in two steps in an age- and activity-dependent fashion: First, vestibular-on-head circuits are reinforced, resulting in head rotation upon otolithic stimulation and reducing undifferentiated movements such as those incorporating ventroflexion. Second, as vestibulospinal pathways mature in the second and third postnatal weeks (10 Donkelaar, 2000), the frequency of immature righting tactics decreases in favour of axial righting. These data strongly suggest that while the animals were flown in space, the maturation of CNS circuits underlying the preference of using one surface righting tactic over another did not proceed as it did in the control animals. Furthermore, in animals from the 16-day mission, such lack of maturation extended for the life of the animal in the presence of otherwise normal vestibular function. Indeed, a recent study has demonstrated changes in descending input and electrical properties of lumbar motoneurons of rats reared in hypergravity (Brocard *et al.* 2003).

The critical period hypothesis

Since surface righting did not take place during the space flight, one may say that the animals were deprived of surface righting in the same way one says that animals are deprived of visual stimuli during a period of lid suture in studies of visual development. The three relevant factors in this respect are the age of the neonates at launch, their age at landing, and mission duration. These are summarized in Fig. 10 for the 16-day mission (Fig. 10A) and the 9-day mission (Fig. 10B). Each panel is divided into a preflight, in-flight, and postflight period. The in-flight and postflight periods are subdivided to represent development

of surface righting tactics (top bar) and speed (middle bar) in flight, and both parameters in control (bottom bar) animals. Periods of development are grey, those periods when development does not take place are white, and those during which adult characteristics are maintained are black. Our data do not allow us to determine when adult values are reached, although they were present at P24 in control animals (Fig. 4B). The findings that inform most directly about sensitive and critical periods of development are those concerning surface righting tactics after the animals returned from space flight.

Our hypothesis proposes: (a) that the maturation of righting tactics involves activity-dependent circuit optimization; (b) that this was arrested in the microgravity environment; and (c) it would not resume after landing if a critical period of development had elapsed. (The first two parts of this hypothesis have been discussed.) It follows that, with respect to this motor synergy, the functional age of the flight animals at launch and landing would be identical. If development resumed after the 9-day mission, axial righting would predominate by P16 (i.e. the first day after landing) and reach adult levels soon after. This was in fact the case (Fig. 4B). The functional age of the animals on the 16-day mission would be P14 at landing, not P30, and the frequency of axial righting would be expected to increase postflight. This was not the case (Fig. 4A). We seek the simplest hypothesis for this last finding.

A possible explanation lies in the animal's chronological age. P30 animals are too old to refine their surface righting tactics and remain functionally P14 for the rest of their

lives. Alternatively, it could be that they were in space too long. Accordingly, 16 days in space, at any age, would have the same effect. Both explanations are consistent with the existence of a critical period of development for the acquisition of surface righting tactics and with the fact that this period elapsed while the animals were in space. Further experiments are needed to find if this is indeed the case.

Role of the peripheral nervous system and the musculoskeletal system

Alternative interpretations of our data include changes in muscle or the peripheral vestibular sensors. Indeed, concerning musculature it is well known that space flight leads to muscle atrophy (Fitts *et al.* 2000, 2001). Studies of neonates on the 16-day mission found increased fast and decreased slow muscle fibre gene expression in hind-limb muscles of flight animals (Adams *et al.* 2000a,b). However, lack of surface righting in microgravity was not due to lack of maturation of the underlying muscular system, as the animals were observed to lack surface righting very shortly after attaining a stable orbit, and so, there was hardly any time for atrophy. This interpretation is consistent with the finding of Trune (Trune & Lim, 1983) that otoconia-deficient mice lack the air-righting reflex, and with the report that mice lacking otoconia have difficulty with balance, even when they are still (Kozel *et al.* 1998). Since surface righting was not tested in these mutant mice, it is not clear whether tactile stimulation would

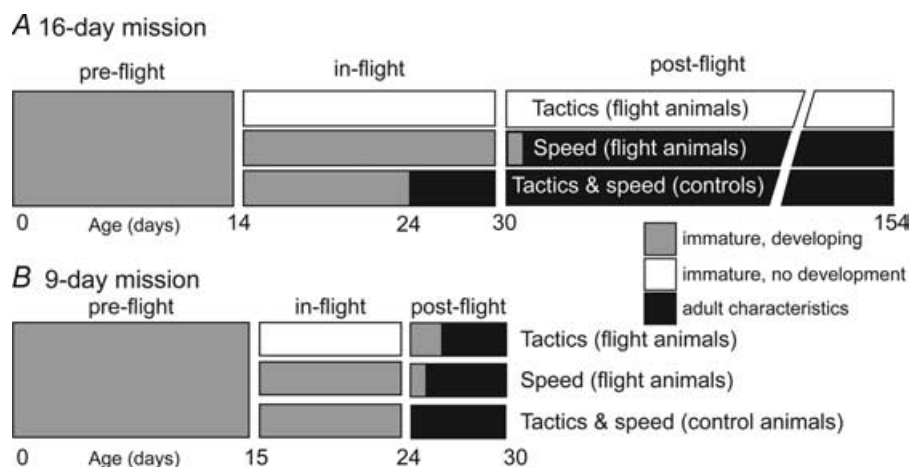


Figure 10. Summary of the effects of microgravity on the mechanisms underlying the maturation of surface righting tactics and surface righting speed for 9-day mission and 16-day mission

A, 16-day mission. Before P14 all animals developed normally (0–14 days, grey). From the time of launch on P14 the mechanism underlying the choice of righting tactics did not continue to mature in the flight animals (top bar, white), this state continued after landing (30–154 days). Mechanisms underlying the speed of righting continued to mature during and after the mission (middle bar, grey) reaching control levels one day after landing (middle bar, black). Maturation continued in control animals (bottom bar, grey) reaching adult levels by P24 (bottom bar, black). **B**, 9-day mission. A similar pattern as above was seen before and during this mission with one exception. Control surface righting tactics were achieved two days after landing (15–30 days, middle bar).

have elicited righting as has been reported for bilaterally labyrinthectomized rabbits (Magnus, 1924).

Although limb muscle strength may influence the movement itself once it has begun, two observations argue against a role in determining surface righting tactics: (1) in flight animals the proportion of axial righting (Figs 4 and 5) and its speed (Fig. 8) were indistinguishable from launch values one day after landing. Muscle atrophy does not recover this quickly; (2) tactics are strongly influenced by head movement in both control and flight animals (Fig. 6). It is unlikely that this is determined by limb musculoskeletal factors. In considering the axial musculature, an increased strength of the external oblique muscle (used in torso rotation) has been found in pregnant adult rats postflight (Fejtek & Wassersug, 1999, 2001). According to this view, the increased abdominal strength would favour non-axial righting tactics. However, the reduction or elimination of righting with ventroflexion from the head-rotated position on landing day (Fig. 6) points to head position as being a key element. Also, corkscrew righting, which incorporates torso rotation, was reduced, not increased postflight (Fig. 5).

Flight animals were able to right themselves on the first postflight trial indicating that the peripheral sensory and motor components required for this movement were functionally intact, as was the motor intention and related strategy for surface righting. Little is known of the changes that take place in the nervous system in animals reared in microgravity, and further studies are needed. With respect to the efferent vestibular network, Raymond's group (Dememes *et al.* 2001) has found that development of this network was not sensitive to microgravity between P8 and P24 (16-day mission). Since the animals in our experiments were in microgravity at a later age, it is unlikely that alterations in development of the efferent vestibular system underlie our findings.

Behavioural studies are limited in that they report what animals do under particular circumstances. They do not directly speak to underlying mechanisms. Thus, our hypotheses relating to the contribution of skeletal, muscular, central and peripheral neuronal elements to the behaviours we have observed must be examined directly in further experiments.

Structural basis for behavioural findings

The ability of newborn rats to right themselves (Pellis *et al.* 1991) indicates that the structural basis for surface righting is present at birth as shown by morphological studies (Lakke, 1997; deBoer-van Huizen & ten Donkelaar, 1999; Dememes *et al.* 2001). In parallel with the emergence of mature motor function, profound changes occur in the structure of the nervous system in the first month after birth at the level of spinal motoneurons (Cummings & Stelzner, 1984; Kalb, 1994), corticospinal circuits (Donatelle, 1977; Uozumi *et al.* 1988; Gianino *et al.*

1999), vestibular circuits (Geisler *et al.* 2000), and the cerebellum (Crepel *et al.* 1976; Kalenga *et al.* 1995; Morara *et al.* 2001). These and other postnatal changes in the structure of the developing nervous system are influenced by the activity of young animals as they interact with their environment (Kalb & Hockfield, 1988; Katz & Shatz, 1996; Penn & Shatz, 1999). Indeed, structural changes have been found in the 16-day mission flight animals at the level of the cervical spinal cord (Inglis *et al.* 2000) and cerebral cortex (DeFelipe *et al.* 2002). Such alterations in synaptic connectivity provide likely candidates for future studies of the morphological correlates of the behavioural observations reported here.

In short, then, the results identify the environmental conditions needed for the development of a motor sequence that incorporates postural intentionality and tactical sequences involving vestibular system function. The sensitivity of the development of the surface righting reaction to the presence of gravity demonstrates that the nervous system does not presuppose the existence of a particular gravitational field. Rather, the unexpected conclusion is that contextual interaction with the environment sculpts the postnatal development of motor function as animals form an internal representation of the world in which they live. This is remarkable given that gravity has been one of the most consistent parameters throughout evolution on this planet.

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Supplemental material

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